

# Evaluation of H3 Histone Methylation and Colony Formation in Erythroid Progenitors Treated with Thalidomide and Sodium Butyrate

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## ABSTRACT

**Objectives:**  $\beta$ -thalassemia and sickle cell disease are hemoglobinopathies with reduced/absent  $\beta$  chains in the former and dysfunctional  $\beta$  chains in the latter. In both conditions, up-regulation of hemoglobin F through demethylation can alleviate the symptoms. This can be attained with drugs such as thalidomide and sodium butyrate.

**Materials and Methods:** This study was performed on erythroid progenitors derived from CD133+ cord blood stem cells. Erythroid progenitors were treated with thalidomide and sodium butyrate in single and combined groups. Colony-formation potential in each group was evaluated by the colony assay. Real-time polymerase chain reaction (RT-PCR) was used to evaluate the effect of these drugs on histone H3 lysine 27 (H3K27) methylation patterns.

**Findings:** Compared to other treatment groups, CD133+ cells treated with thalidomide alone produced more hematopoietic colonies. Thalidomide alone was also more effective in decreasing H3K27 methylation.

**Conclusions:** Thalidomide shows superiority to sodium butyrate as a hypomethylating agent in this cell culture study, and it has the potential to become conventional treatment for sickle cell disease and  $\beta$ -thalassemia.

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**KEY WORDS:**  $\gamma$ -globin gene • Epigenetic induction • Thalidomide • Sodium butyrate • H3K27 methylation

## INTRODUCTION

Human  $\beta$ -globin locus is located on the short arm of chromosome 11 in a cluster of 5 active genes including  $\epsilon$ ,  $\gamma_G$ ,  $\gamma_A$ ,  $\delta$ , and  $\beta$ .  $\epsilon$ -globin gene expression is limited to the first trimester.  $\gamma_G$  and  $\gamma_A$  gene expression is active throughout the fetal life, and  $\delta$ -globin and  $\beta$ -globin genes are mostly expressed postpartum [1].  $\beta$ -globin locus switching occurs in 2 steps: silencing of  $\epsilon$ -globin gene in the embryonic stage accompanied by  $\gamma_G$  and  $\gamma_A$  gene activation, followed by a gradual deactivation of  $\gamma_G$  and  $\gamma_A$  genes and a progressive increase in  $\delta$ - and  $\beta$ -globin expression [2].

Induction of  $\gamma_G$  and  $\gamma_A$  globin genes and consequently elevation of postnatal fetal hemoglobin (HbF) could be considered an efficient therapeutic strategy in treatment of  $\beta$ -thalassemia and sickle cell disease (SCD). HbF induction in these patients decreases formation of excessive  $\gamma$ -globin chains in  $\beta$ -thalassemia and hemoglobin polymers in SCD. This will ameliorate the symptoms in both conditions [3,4]. In recent years, the use of drugs that alter the epigenetic patterns has increased as a way to affect gene expression. Azacytidine, decitabine, butyrate, pamalidomide, and thalidomide are among some of the drugs with HbF-inducing potential [5-9]. Some of these drugs increase the expression of HbF through reducing the methylation of specific CpG (—C—phosphate—G—) regions located upstream of  $\gamma_G$  and  $\gamma_A$  genes. Others can provide this effect through histone acetylation, especially H3 and H4 acetylation

at promoter of  $\gamma$ -globin gene [10,11]. It has been seen that alterations in histone methylation patterns can also affect gene expression. Methylation of histone H3 lysine 4 (H3K4) and H3K36 is observed in the euchromatin, and methylation of histone H3 lysine 27 (H3K27) and H4 is the usual pattern found in the heterochromatin. Recent studies suggest that trimethylated H3K9 and H3K27 exist in the coding regions of activated globin genes [11].

In the present study, we focus on the H3K27 methylation pattern in erythroid progenitors. Methylation of this amino acid is usually marked by gene inactivity. Elucidating its methylation pattern and the effects of its demethylation may therefore help find effective ways to up-regulate HbF.

## METHODS AND MATERIALS

### CD133+ Stem Cell Isolation

CD133+ stem cells were isolated from human cord blood. Consent was obtained from 3 healthy pregnant women, and cord blood was collected from the umbilical cords immediately after delivery. To isolate mononuclear cells (MNCs), 100 mL of cord blood was diluted with hydroxyl ethyl starch (HES) in a ratio of 1 to 6. Then cells were layered onto 15 mL of Ficoll-Paque (Amersham Pharmacia, Piscataway, NJ, USA;  $\rho = 1.077$  g/mL). Centrifugation was carried out at 400 g for 40 minutes. After centrifugation, the MNC layer was collected and diluted in 3 volumes of phosphate buffered saline (PBS)–ethylenediaminetetraacetic acid (EDTA) (pH = 7.2) and centrifuged at 300 g for 10 minutes. This was repeated 3 times, and the final pellet was re-suspended in PBS/EDTA (pH = 7.2). CD133+ hematopoietic stem cells were separated from MNCs by the MACS® technique (Miltenyi Biotec, Bergisch Gladbach, Germany). In short, 100  $\mu$ L of CD133+ microbeads were added to MNCs and incubated for 30 minutes at 4°C and centrifuged for 7 minutes at 400 g. Next, the supernatant was removed, and the PBS/EDTA (pH = 7.2) was added to the pellet. Finally, cells were passed through the LS Column (Miltenyi Biotec) according to the manufacturer's instruction. Then, 5  $\times 10^4$  cells were cultured in 24-well plates. Cells were expanded for 1 week in StemSpan® media (STEMCELL Technologies Inc., Vancouver, BC, Canada) supplemented with StemSpan® CC110 (stem cell factor [SCF], trombopoietine [TPO], Fms-like Tyrosine Kinase-3 [FLT-3] ligand cocktail; STEMCELL Technologies Inc.).

### Flow Cytometry Analysis

Flow cytometry was performed to evaluate the homogeneity and purity of isolated and expanded CD133+ stem cells. For this purpose, 100  $\mu$ L of PBS was added to 1  $\times 10^4$  cells. Then, 7  $\mu$ L of phycoerythrin (PE) labeled anti-CD133 (PE; clone AC141; Miltenyi Biotec) monoclonal antibody was added to cells. The suspension was incubated at 4°C in the dark for 60 minutes. Cells were fixed with 1% paraformal-

hyde (PFA; Invitrogen, Carlsbad, CA, USA). Mouse fluorescein isothiocyanate (FITC)-labeled immunoglobulin (Ig) G1 (IQP-191F, IQ Products, Groningen, Netherlands) was used as isotype control.

### Colony-Forming Assay

In order to assess the effects of thalidomide and sodium butyrate on the hematopoietic ability of CD133+ stem cells as well as their capability to differentiate into erythroid lineage, the colony assay was employed. CD133+ cells (2  $\times 10^3$ /mL) were cultured in MethoCult medium (MethoCult H4230, STEMCELL Technologies Inc.), which contains Iscove's Modified Dulbecco's Medium (IMDM) with 2% fetal bovine serum (FBS) (Cambrex Bio Science Verviers, Verviers, Belgium), 5 ng/mL IL-3, and 3 U/mL erythropoietin in 35-mm petri dishes. The study was performed in 4 groups as follows: 100  $\mu$ M thalidomide (100T); 100  $\mu$ M sodium butyrate (100S); 100  $\mu$ M thalidomide + 100  $\mu$ M sodium butyrate (100 T/S); and 0.1% dimethylsulfoxide (DMSO) as negative control. The dishes were incubated at 37°C and 5% CO<sub>2</sub> for 14 days. After 14 days, a benzidine stain was added to petri dishes for 15 minutes. A solution containing 50% methanol, 50% distilled water, and 1 mL H<sub>2</sub>O<sub>2</sub> was then added and incubated for 20 minutes. Finally, the total number of colonies and benzidine-positive colonies were counted by inverted microscope.

### Differentiation into Erythroid Progenitors

To differentiate purified CD133+ cells into erythroid progenitors, cells were cultured in IMDM (Gibco®, Invitrogen) supplemented with 30% FBS (Cambrex Bio Sciences Verviers), 70  $\mu$ g/mL iron-saturated transferrin, 2 mM L-Glutamine (Invitrogen), 10  $\mu$ M 2-mercaptoethanol (2-ME; Sigma-Aldrich, Saint Louis, MO, USA), 100 U/mL penicillin/Streptomycin (Gibco®, Invitrogen), 3 U/mL human recombinant erythropoietin (R&D Systems, Minneapolis, MN, USA) and 5 ng/mL IL-3 (STEMCELL Technology Inc.). After 7 days, the newly formed erythroid progenitors were treated with sodium butyrate (Sigma-Aldrich) and thalidomide (Tocris Bioscience, Ellisville, MO, USA). The study was performed in 4 drug groups as previously mentioned. Media was changed every 3 days. After 14 days, cells were harvested.

### Chromatin Immunoprecipitation (ChIP)

To study protein–gene interactions and histone modification of DNA regions of interest, ChIP was carried out. In brief and according to manufacturer's protocols (EpiQuik Methyl-Histone H3K27 ChIP Kit; Epigentek, Farmingdale, NY, USA), approximately 5  $\times 10^6$  cells were collected from different groups of study. Cells were lysed, and cellular chromatin was fragmented by sonication technique. Chromatin fragments were then added to the bottom of wells previously coated with antibodies against tri-methylated histone H3 at lysine 27 (H3K27me<sub>3</sub>). Chromatin fragments that contain

TABLE 1. Admission Laboratory Data

d	Forward Primer	Reverse Primer
$\gamma$ -globin	GGCTGGCTAGGGATGAAGAATAAA	TGGCGTCTGGACTAGGAGCTTA
$\beta$ -actin	CCCTGGCGGCCTAAGGACTC	CACATGCCGGAGCCGTTGTC

methylated lysine bind to wells, and other fragments are removed from the bottom of wells by washing. Next, precipitated chromatin fragments were detached from antibodies, and DNA fragments were separated by reverse cross-link technique. Eventually, DNA fragments were purified.

### Quantitative Analysis of Histone Methylation Modification

In order to evaluate and quantitatively compare DNA fragments from different groups (purified by the ChIP technique), SYBR Green real-time polymerase chain reaction (RT-PCR) (Qiagen Kit, QIAGEN, Valencia, CA, USA) was performed with specific primers for  $\gamma$ -globin promoter. The samples were amplified using Applied Biosystems' "StepOne Real-Time PCR System" (Applied Biosystems Inc., Foster City, CA, USA). The table shows primers designed for genomic  $\gamma$ -globin and  $\beta$ -actin for ChIP-RT-PCR analysis. The results were normalized using  $\beta$ -actin as the internal control. Relative quantity was determined using the comparative CT method and calculated by the  $\Delta$ Ct. The data were analyzed by the formula  $2^{-\Delta\Delta Ct}$ .

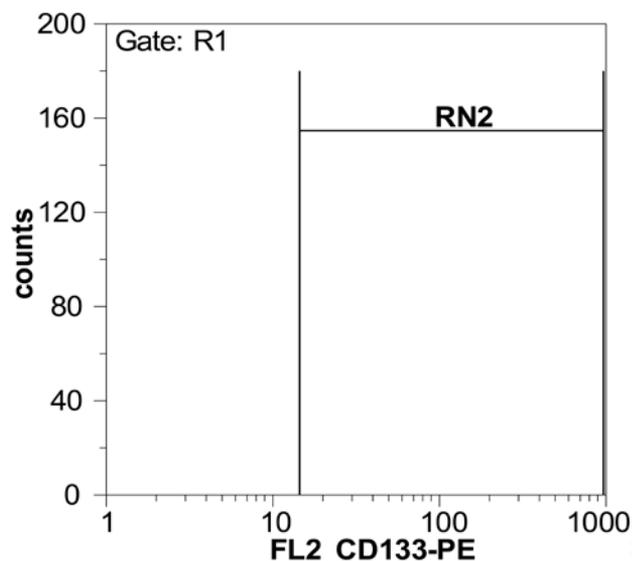


Figure 1. Homogeneity of CD133+ cells after 7 days of expansion. Flow cytometry analysis of at least 1  $\times 10^4$  cells showed that more than 95% of cells were presenting the CD133 marker. RN2 is outlined for gating cells that are in fluorescence channel (FL2) that phycoerythrin (PE) is detected in this channel.

### Statistical Analysis

Statistical analysis and graph preparations were performed using Microsoft Excel 2007 (Microsoft, Redmond, WA, USA) and SPSS software (IBM, Armonk, NY, USA). The *P*-value of less than .05 was considered statistically significant for *t* test analysis.

## RESULTS

### Flow Cytometry Analysis

Seven days after expansion of isolated CD133+ stem cells, flow cytometry was used to evaluate cell homogeneity. The analysis showed a purity of approximately 95% for isolated CD133+ stem cells after expansion (Figure 1). The data suggest that the expansion process has resulted in proliferation of CD133+ stem cells without differentiation into more mature lineages. Because purity of isolated and expanded cells for providing effective erythroid differentiation is necessary, our research was performed on pure CD133+ stem cells.

### Effect of Thalidomide, Sodium Butyrate, and Combination Treatment on Colony Formation

Results from total hematopoietic colony count and benzidine-positive colonies (erythroid colonies) in defined group are shown in Figure 2. Our results demonstrated that thalidomide was more effective in increasing the number of total hematopoietic colonies including erythroid and non-erythroid colonies as well as erythroid colonies alone in comparison to sodium butyrate and combination treatment. We showed that thalidomide increased total hematopoietic colony number and erythroid colony number by 1.43-fold and 1.88-fold, respectively, compared to DMSO as negative control. Sodium butyrate increased total hematopoietic and erythroid colony numbers by 1.09-fold and 1.27-fold, respectively, compared to negative control. Also, the combined effect of thalidomide and sodium butyrate on total hematopoietic and erythroid colony numbers was less than thalidomide alone. Compared to negative control, the combined group increased the total hematopoietic and erythroid colony numbers by 1.21-fold and 1.38-fold, respectively. All differences were statistically significant ( $P < .05$ ).

### Effects of Thalidomide, Sodium Butyrate, and Combination Treatment on H3 Histone Methylation Pattern in $\gamma$ -Globin Gene

To assess the epigenetic mechanisms of HbF-inducing drugs, we investigated the methylation modification pattern in H3K27 following the treatment of erythroid progenitors with thalidomide and sodium butyrate alone and combined.

### Hematopoietic colony formation assay

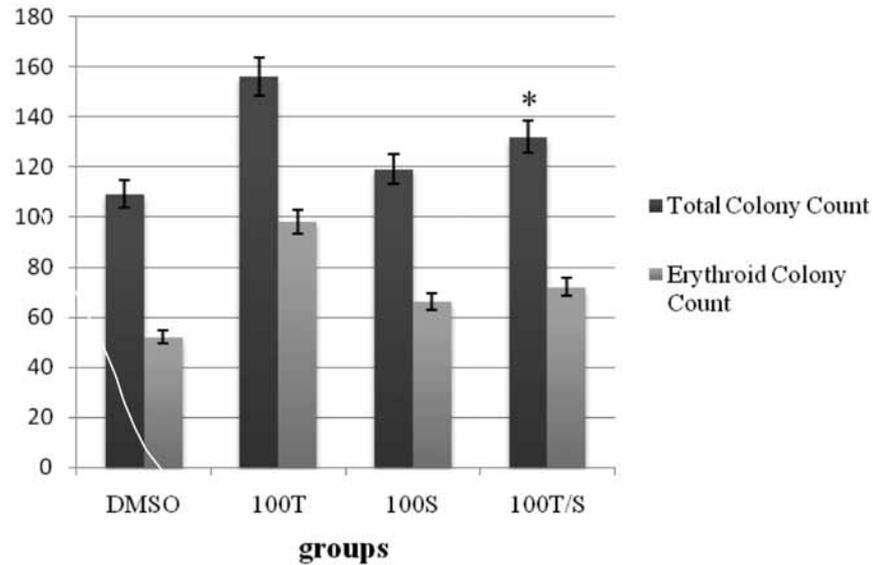


Figure 2. The effect of thalidomide, sodium butyrate, and combined treatments on the colony count. CD133+ cells isolated from umbilical cord blood were cultured in methylcellulose media containing erythropoietin (EPO) and interleukin (IL)-3. After 14 days, all colonies including the erythroid colonies (benzidine positive) were counted. Results are shown as mean ( $\pm$  standard deviation) from 3 different samples ( $P < .05$ ).

### H3K27 Enrichment

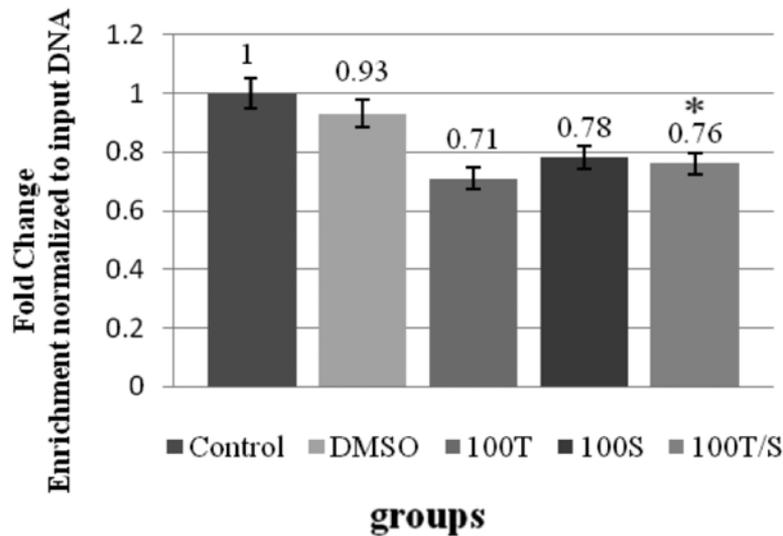


Figure 3. Thalidomide (T), sodium butyrate (S) alone, and T/S combined regulate histone H3 lysine 27 (H3K27) methylation on  $\gamma$ -globin genes during erythroid differentiation. After 14 days of erythroid differentiation in the presence of negative control (dimethylsulfoxide [DMSO]), treated progenitor cells were subjected to chromatin immunoprecipitation (ChIP) using anti-tri-methylated histone H3 at lysine 27 (H3K27me3) antibody and analyzed by quantitative real-time polymerase chain reaction (RT-PCR) using primers specific for  $\gamma$ -globin genes. Results are shown as mean ( $\pm$  standard deviation) from 3 different samples. The thalidomide group has the lowest copy numbers of methylated DNA ( $P < .05$ ).

As shown in Figure 3, the methylation modification pattern of H3K27 showed a 0.71-fold decrease in thalidomide group compared to DMSO as negative control ( $P < .05$ ). The decrease was 0.78-fold and 0.76-fold in sodium butyrate and in combined treatment group, respectively ( $P < .05$ ). These data showed that the decrease in H3K27 methylation was highest in the thalidomide group.

## DISCUSSION

We focused on the H3K27 histone methylation pattern following the treatment of erythroid progenitors with thalidomide and sodium butyrate in defined groups (100T, 100S, 100 T/S, and DMSO). Our results confirmed previous studies on reduction of H3K27 methylation in  $\gamma$ -globin gene [10,11]. We assume that the better efficiency in demethylating histones with thalidomide compared to sodium butyrate can possibly make it more effective in up-regulation of HbF. To delineate this, we suggest that further studies are carried out on measuring and comparing HbF in the presence of both drugs.

Our findings also revealed that thalidomide by itself was significantly more effective in reducing H3K27 methylation. We do not know the exact reason behind this. However, the following explanations may justify this observation: 1) Thalidomide is inherently better than sodium butyrate in demethylating H3K27. 2) Sodium butyrate and thalidomide were used at the same concentrations. In higher concentrations, however, sodium butyrate may be more potent in demethylating histones. If so, this will make thalidomide more preferable because sodium butyrate is more toxic in higher doses. 3) Both drugs are competing for methyl groups; one may inhibit the other, and this may result in less demethylation observed in the combined group.

In corroboration with the findings of Aerbajinai and coworkers [9], our results from hematopoietic colony-forming assay showed that CD133+ can produce more colonies, including erythroid colonies, in the presence of thalidomide alone. This indicates that thalidomide has less cytotoxic effects on proliferation and expansion compared to sodium butyrate. Sodium butyrate has mild cytotoxic effects, and this may account for the lower colony numbers in this group [4]. As shown in Figure 2, the majority of hematopoietic colonies are erythroid colonies clarified the effect of both thalidomide and sodium butyrate on erythroid colony formation. This finding has strong implications in clinical settings where toxicity is a major issue in administering drugs. Our research was based on using nontoxic concentrations of both thalidomide and sodium butyrate. As mentioned, we used 100  $\mu$ M of drug concentrations in defined groups of cell culture, and the concentrations up to 100  $\mu$ M for thalidomide and 150  $\mu$ M for sodium butyrate seem to be nontoxic effects because they have no inhibitory effect on colony formation and cell growth [7,9].

All in all, the lower toxicity and possibly the higher HbF-producing potential make thalidomide a better candidate for the treatment of SCD and  $\beta$ -thalassemia.

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